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Loss of Erythrocyte Deformability under Oxidative Stress is caused by Protein Oxidation with Consequent Degradation Rather than by Lipid Peroxidation

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

Aims: Loss of erythrocyte deformability under oxidative stress is poorly understood. The present study aimed to determine which of the detrimental effects of oxidant stress, namely, lipid peroxidation or protein degradation, is responsible for loss of erythrocyte deformability.
Methodology: Different natural and synthetic antioxidants were tested for their protective effects on erythrocyte deformability, lipid peroxidation and protein degradation after exposure to H_2O_2. Antioxidants used included α-Tocopherol (vitamin E), Butylated Hydroxytoluene (BHT), vitamin C, PNU-101033E, carbon monoxide (CO) and selected flavonoids and herbal extracts.
Results: Exposure of human erythrocytes *in vitro* to H_2O_2 caused loss of deformability, lipid peroxidation and protein degradation. Pre-incubation of erythrocytes with vitamin E, BHT, vitamin C, PNU-101033E, the flavonoids rutin and morin and herbal extracts of *Ferula hermonis*, *Hibiscus sabdariffa*, *Teucrium polium*, prevented lipid peroxidation caused by H_2O_2 but did not prevent loss of erythrocyte deformability, nor protein degradation. CO, the flavonoid quercetin and herbal extracts of *Nigella sativa* and *Allium sativum* prevented both lipid peroxidation and protein degradation, but also prevented loss of erythrocyte deformability. The flavonoid 3,5,7-trihydroxy-4'-

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methoxy flavone-7-rutinoside prevented both protein degradation and loss of deformability, with no effect on lipid peroxidation. Vitamin C, unexpectedly, caused a significant increase in loss of erythrocyte deformability induced by H_2O_2 in parallel to the increased rate of protein degradation.
Conclusion: These results suggest that protein degradation rather than lipid peroxidation is responsible for loss of erythrocyte deformability under oxidative stress. Also that lipid peroxidation and protein degradation occur by independent mechanisms. This study should initiate a search for potential drugs that can prevent protein oxidation as well as lipid peroxidation, thereby acting in the prevention of adverse hemorheological consequences in disease states associated with oxidative stress. Caution should be exercised in the therapeutic use of vitamin C, especially under oxidant stress.

Keywords: Erythrocyte deformability; oxidative stress; lipid peroxidation; protein oxidation; Vitamin C; antioxidants; protein degradation; alanine; MDA.

1. INTRODUCTION

Deformability is the basic rheological property of the erythrocyte. Erythrocyte deformability, as one of the main determinants of blood flow in macr- and micro-circulation, is the ability of the erythrocyte to undergo deformation when subjected to shear stress, which is necessary for blood flow in the microcirculation, allowing erythrocytes to pass through vessels as narrow as 2-3 µm in diameter [1,2]. *In vivo* erythrocytes are constantly exposed to intracellular and extracellular oxygen radicals and whose direct measurement is difficult to make, necessitating indirect measurement of degradation products, namely, lipid peroxidation and protein products [3]. Malonyldialdehyde (MDA) and alanine are widely used as indicators of lipid peroxidation and protein degradation, respectively. Exposure of erythrocytes to certain chemical reactions, which can generate oxygen free radicals, can lead to erythrocyte damage consequent upon lipid peroxidation and protein degradation with disturbance of membrane permeability [4,5]. Such findings can help explain infection-mediated hemolysis in sickle cell anemia and glucose-6-phosphate-dehydrogenase deficiency [6]. Previous studies from our laboratory showed that exposure of erythrocytes to oxygen radicals caused lipid peroxidation, protein degradation and loss of deformability [7,8]. Those studies, however, were unable to determine the real cause of loss of erythrocyte deformability, namely, whether loss of deformability was due to lipid peroxidation alone, protein degradation alone or due to the cumulative effect of both. To achieve this goal, we selected the following natural and synthetic antioxidants: α-Tocopherol (vitamin E), Butylated Hydroxytoluene (BHT), vitamin C, PNU-101033E (a potent inhibitor of lipid peroxidation developed by Pharmacia & Upjohn), carbon monoxide (CO) and selected flavonoids and herbal extracts to study their effects on deformability, lipid peroxidation and protein degradation of human erythrocytes exposed to H_2O_2.

2. MATERIALS AND METHODS

2.1 Flavonoids and Herbal Material

The following flavonoids (quercetin, 3,5,7-trihydroxy-4'-methoxy flavone-7-rutinoside, rutin and morin) were purchased from All Aldrich Chemical Company, Milwaukee, USA. The following herbal material (seeds of *Nigella sativa*, bulb of *Allium sativum*, roots of *Ferula hermonis*, calyx of *Hibiscus sabdariffa*, leaves of *Teucrium polium,* seeds of *Trigonella foenum-graecum* and leaves of *Artemisia herba-alba* were purchased from the local market and identified by a taxonomist at the University of Jordan.

2.2 Preparation of Herbal Extracts

The methanolic extracts of the tested herbal material were prepared as described elsewhere [9].

2.3 Exposure of Erythrocytes to H_2O_2 with and Without Antioxidant

Leukocyte-depleted and platelet-depleted pure erythrocyte suspensions were used for all experiments, prepared by pre-filtration of heparinized whole blood from adult volunteers through Imugard IG500 cotton wool (Termo Corp., Tokyo, Japan) [10]. Filtered erythrocytes were washed three times and resuspended in phosphate buffered saline (PBS; 290±5 mOsmol/Kg water, pH=7.40±0.02) at PCV values adjusted to 14% for deformability studies and 5% for MDA and alanine determination studies. Pure

erythrocyte suspensions were pre-incubated with sodium azide (2 mM), to inactivate catalase and myeloperoxidase enzymes [11] for 30 min at 37ºC in a shaking water-bath and then incubated with a given antioxidant for further 30 min, followed by incubation with H_2O_2 (10mM) for 60 min in the same shaking water-bath. Buffer controls contained buffer instead of H_2O_2 and stress controls contained H_2O_2 alone without antioxidant. After incubation, suspensions were mixed and used for deformability, MDA and alanine measurements, respectively.

2.4 Erythrocyte Deformability Determination

Erythrocyte deformability was determined by filtration of erythrocyte suspension through 5μm pores diameter polycarbonate membranes (Nuclepore corporation, Pleasanton, USA) using a temperature controlled Hemorheometer MK1 (St. Witz, France) [12]. A small batch of 10 membranes were used and reused after cleaning by ultrasonication in aqueous sodium dodecyl sulfate (1%, w/v) for 10 seconds [13]. Results were expressed as an index of filtration (IF) of the flow time for the erythrocyte suspension relative to buffer and corrected for hematocrit [10]. An increase in IF indicates loss of filterability (deformability), and vices versa. Filtration result (IF) for each antioxidant with H_2O_2 was compared with those for stress control erythrocytes treated similarly but without the antioxidant (i.e., with H_2O_2 alone).

2.5 Erythrocyte MDA Determination

MDA was determined as a measure of lipid peroxidation according to Stock's and Dormandy's method [14] as modified by others [15]. All MDA concentrations were expressed as nmol/gHb.

2.6 Erythrocyte Alanine Determination

Alanine is not synthesized *de novo* in erythrocytes, so net production of alanine can only occur via protein degradation. Alanine was determined as a measure of protein degradation according to Davies and Goldberg [4] as modified by others [7]. All alanine concentrations were expressed in nmol/gHb.

2.7 Statistical Analysis

Data were presented as mean±SD. Statistical significance was determined using one-way analysis of variance followed by student t-test for paired samples, using SPSS version 17. Differences were considered significant when $p \leq 0.05$.

3. RESULTS

3.1 Effects of Vitamin E and BHT

Erythrocytes pre-incubated with vitamin E (0.34 mM) and then exposed to H_2O_2 showed no significant change in erythrocyte IF (i.e. no change in deformability) or alanine production (i.e. no change in protein oxidation), compared to control erythrocytes exposed to H_2O_2 alone (Fig. 1). However, it showed a significant inhibition of MDA production from a mean of 293.2 nmol/g Hb (with H_2O_2 alone) to a mean of 137.8 nmol/g Hb (with H_2O_2 plus vitamin E) (*i.e.* anti-lipid peroxidation) (Fig. 1).

Erythrocytes pre-incubated with BHT (O.2mM) and then exposed to H_2O_2 showed no significant change in erythrocyte IF (i.e. no change in deformability) or alanine production (i.e. no change in protein oxidation), compared to control erythrocytes exposed to H_2O_2 alone (Fig.2). However, it showed a significant inhibition of MDA production from a mean of 343.6 nmol/g Hb (with H_2O_2 alone) to a mean of 145.2 nmol/g Hb (with H_2O_2 plus BHT) (*i.e.*, anti-lipid peroxidation) (Fig. 2).

3.2 Effects of Vitamin C

Erythrocytes pre-incubated with vitamin C (0.2 mM) and then exposed to H_2O_2 showed a significant increase in erythrocyte IF and alanine production, compared to control erythrocytes exposed to H_2O_2 alone (Fig. 3). The IF value increased from a mean of 76.3 (with H_2O_2 alone) to a mean of 90.2 (with H_2O_2 plus vitamin C) (i.e. increased loss of erythrocyte deformability). Alanine production increased from a mean of 4401.0 nmol/g Hb (with H_2O_2 alone) to a mean of 5180 nmol/g Hb (with H_2O_2 plus vitamin C) (i.e. increased protein oxidation). However, *vitamin C* also caused a significant decrease in MDA production. MDA concentration decreased from a mean of 188.0 nmol/g Hb (with H_2O_2 alone) to a mean of 76.7 nmol/g Hb (with H_2O_2 plus vitamin C) (i.e., anti-lipid peroxidation) (Fig. 3).

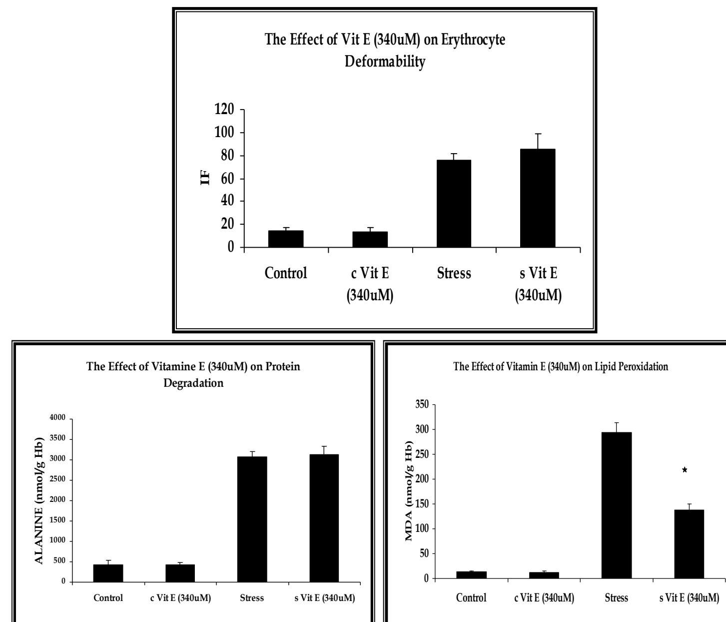

Fig. 1. Index of filtration (IF), Alanine and MDA productions of normal erythrocytes when incubated at 37ºC in buffer alone (control), in buffer containing vitamin E, in buffer containing 10 mM H_2O_2 (stress) and in buffer containing 10 mM H_2O_2 plus pre-incubation with *vitamin E* (0.34 mM). Mean and SD are for eight duplicate experiments representing eight individuals.

** $p < 0.05$ compared with buffer containing H_2O_2 alone*

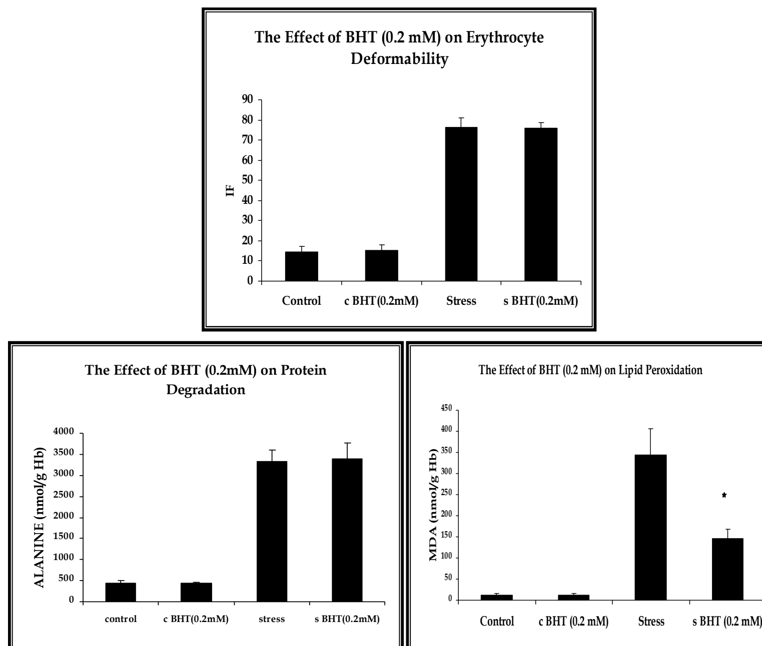

Fig. 2. Index of filtration (IF), Alanine and MDA productions of normal erythrocytes when incubated at 37ºC in buffer alone (control), in buffer containing BHT (O.2 mM) , in buffer containing 10 mM H_2O_2 (stress) and in buffer containing 10 mM H_2O_2 plus pre-incubation with BHT (O.2 mM). Mean and SD are for eight duplicate experiments representing eight individuals.

** $p < 0.05$ compared with buffer containing H_2O_2 alone*

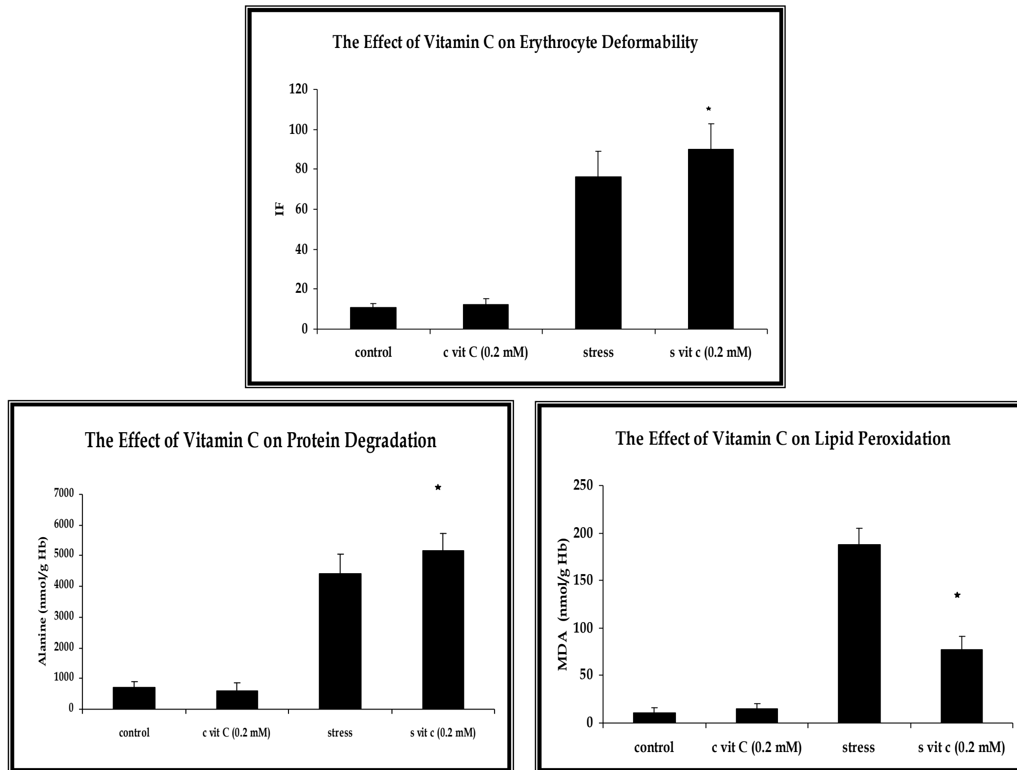

Fig. 3. Index of filtration (IF), Alanine and MDA productions of normal erythrocytes when incubated at 37°C in buffer alone (control), in buffer containing vitamin C (0.2 mM), in buffer containing 10 mM H_2O_2 (stress) and in buffer containing 10 mM H_2O_2 plus pre-incubation with *vitamin C* (0.2 mM). Mean and SD are for eight duplicate experiments representing eight individuals.

** $p < 0.05$ compared with buffer containing H_2O_2 alone*

3.3 Effects of PNU-101033E

Erythrocytes pre-incubated with PNU-101033E at the following concentrations (0.2, 2, 20, 200, 400 or 600 µM) and then exposed to H_2O_2, showed no significant change in erythrocyte IF or in alanine production compared to control erythrocytes exposed to H_2O_2 alone (Fig. 4). However, it showed a significant decrease in MDA production (i.e., decreased lipid peroxidation). As shown in Fig. 4, six different concentrations of PNU-101033E were used (0.2, 2, 20, 200, 400 or 600 µM) and the MDA levels were decreased from a mean of 242.16 nmol/g Hb (with H_2O_2 alone) to the following means (183.25, 154.11, 122.46, 136.86, 128.74, 127.23 nmol/g Hb) respectively with PNU-101033E concentrations.

3.4 Effects of CO

Erythrocytes pre-incubated with *CO* and then exposed to 10 mM H_2O_2 showed almost complete inhibition of the increase in erythrocyte IF. The IF value decreased from a mean of 52.9 (with H_2O_2 alone) to a mean of 18.1 (with H_2O_2 plus CO) (i.e. decreased loss of erythrocyte deformability). At the same time, CO had no effect on IF of control erythrocytes incubated in the absence of H_2O_2. However, *CO* also caused a significant inhibition of alanine and MDA productions. The alanine production decreased from a mean of 2090.31 nmol/g Hb (with H_2O_2 alone) to a mean of 1465.7 nmol/g Hb (with H_2O_2 plus CO) (i.e. decreased protein oxidation), and MDA production decreased from a mean of 287.29 nmol/g Hb (with H_2O_2 alone) to a mean of 187.34 nmol/g Hb (with H_2O_2 plus CO) (i.e., anti-lipid peroxidation) (Fig. 5).

3.5 Effects of Selected Flavonoids

Pre-incubation of erythrocytes with four selected flavonoids (90 mg/ml), only quercetin and 3,5,7-trihydroxy-4'-methoxy flavone-7-rutinoside inhibited significantly the increase in IF

(i.e. decreased loss of deformability) and the increase in alanine (i.e. decreased protein oxidation) of erythrocytes exposed to H2O2, whereas rutin and morin showed no effect on deformability loss caused by H2O2 (i.e. no effect on IF) (Table 1). However, quercetin, rutin and morin caused a significant inhibition of MDA production (i.e., ant-lipid peroxidation), whereas, 3,5,7-trihydroxy-4'-methoxy flavone-7-rutinoside caused no effect on MDA production (Table 1).

3.6 Effects of Selected Herbal Extracts

Pre-incubation of erythrocytes with selected herbal extracts, only *Nigella sativa* and *Allium sativum* inhibited significantly the increase in IF (i.e. decreased loss of deformability), the increase in alanine (i.e. decreased protein oxidation) and the increase in MDA (i.e. anti-lipid peroxidation) of erythrocytes exposed to H_2O_2 (Table 2). The following herbs *Ferula hermonis*, *Hibiscus sabdariffa*, *Teucrium polium*, although inhibited significantly the production of MDA (i.e. anti-lipid peroxidation), they did not inhibit the increase in IF. However, *Trigonella foenum-graecum* unexpectedly increased the production of MDA (i.e. increased lipid peroxidation), but it did not increase the IF. *Artemisia herba-alba* had no effect on MDA or alanine production or IF (Table 2). The beneficial effects of *Nigella sativa* and *Allium sativum* extracts on IF were proportional to their inhibitory effects on alanine production (Fig. 6).

4. DISCUSSION

The oxygen radical generating system of hydrogen peroxide (H_2O_2) was used in the present study. This compound is known to cross the erythrocyte membrane and rapidly reacts with hemoglobin, generating very reactive oxygen radicals with consequent oxidative stress [16].

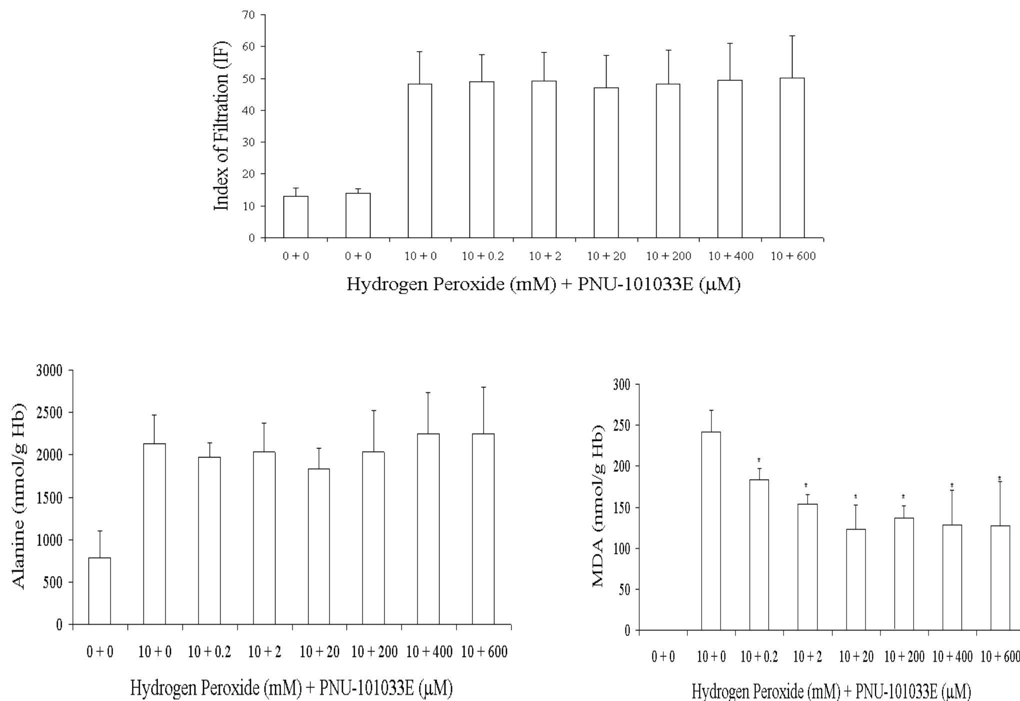

Fig. 4. Index of filtration (IF), Alanine and MDA productions of normal erythrocytes when incubated at 37ºC in buffer alone (0 + 0), in buffer containing ethanol (0 + 0), in buffer containing 10 mM H_2O_2 and ethanol (10 + 0) and in buffer containing 10 mM H_2O_2 and pre-exposure to different concentrations of PNU-101033E (0.2, 2, 20, 200, 400 or 600 μM).

** $p < 0.05$ compared with buffer containing H_2O_2 alone*

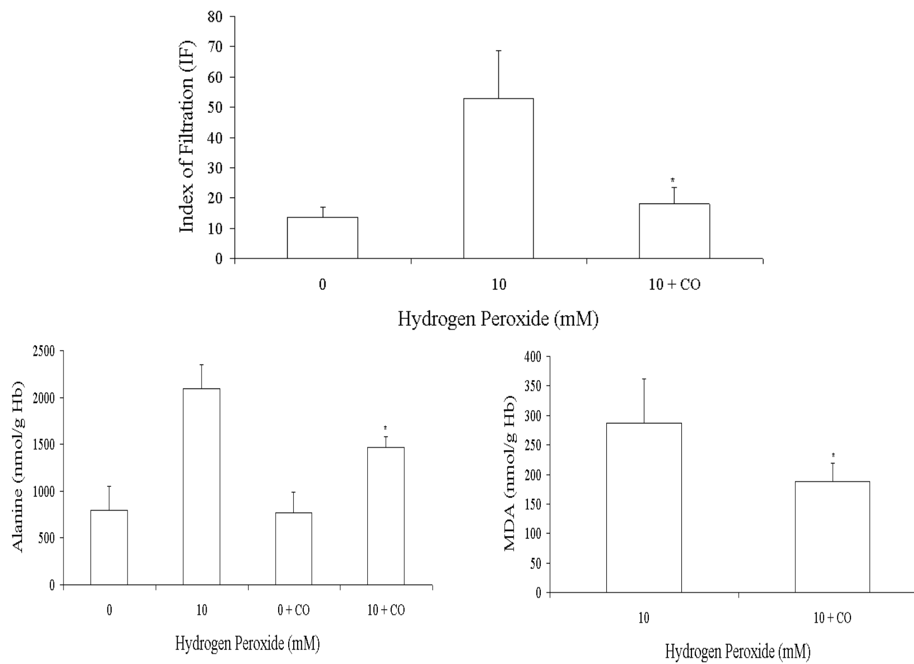

Fig. 5. Index of filtration (IF) of normal erythrocytes when incubated at 37ºC in buffer alone (0), in buffer containing 10 mM H_2O_2 (10) and in buffer containing 10 mM H_2O_2 plus pre-exposure to carbon monoxide gas (10 + CO). Mean and SD are for eight duplicate experiments representing eight individuals.

** $p < 0.05$ compared with buffer containing H_2O_2 alone*

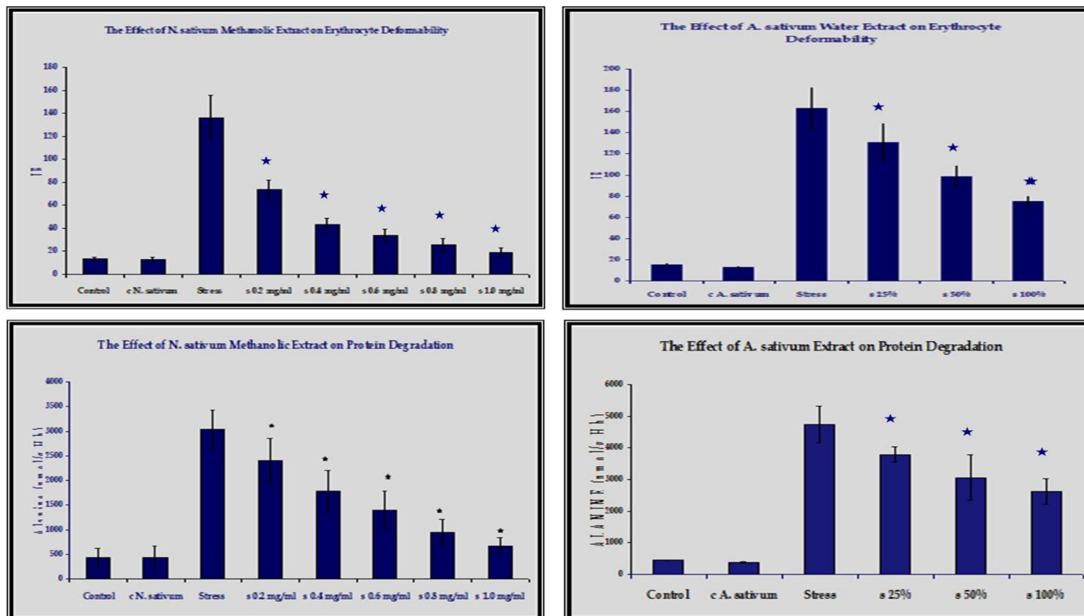

Fig. 6. Index of filtration (IF) and Alanine production of normal erythrocytes when incubated at 37ºC in buffer alone (control), in buffer containing *Nigella sativa* or *Allium sativum* extracts, in buffer containing 10 mM H_2O_2 (stress) and in buffer containing 10 mM H_2O_2 plus pre-incubation with *Nigella sativa* or *Allium sativum* extracts at increasing concentrations. Mean and SD are for eight duplicate experiments representing eight individuals.

** $p < 0.05$ compared with buffer containing H_2O_2 alone*

Table 1. Alanine and MDA concentrations, and IF of normal erythrocytes when incubated at 37ºC for 60 min with or without 10 mM H_2O_2 or with H_2O_2 plus different concentrations of tested flavonoids. Values are presented as a mean ± S.D. of 5 experiments with duplicate tubes (*P < 0.05 - compared to control erythrocytes with H_2O_2 alone)

	Flavonoid	Alanine (nmol/g Hb)		MDA (nmol/g Hb)		IF	
Flavonoid	**Conc. (mg/ml)**	**Without H_2O_2**	**With H_2O_2**	**Without H_2O_2**	**With H_2O_2**	**Without H_2O_2**	**With H_2O_2**
Control	**0**	**519±108**	**3504±280**	**20.0±3.7**	**402.0±56.7**	**11.9±2.4**	**116.4±10.5**
Quercetin	90	501±80	2479±199*	23.0±3.3	195.9±39.5*	11.5±1.8	70.5±8.6*
3,5,7-trihydroxy- 4'-methoxy flavone-7-rutinoside	90	586±87	2950±161*	20.0±9.0	377.7±46.0	11.6±2.4	86.5±12.4*
Rutin	90	505±106	3498±208	21.0±5.4	247.5±33.0*	11.1±1.3	118.3±14.5
Morin	90	510±76	3750±265	23.0±7.0	264.0±45.0*	12.0±2.1	120.3±13.5

Table 2. Alanine and MDA Concentrations and IF of normal erythrocytes when incubated at 37 ºC for 60 min with or without 10 mM H_2O_2 or with H_2O_2 plus different concentrations of tested medicinal plant extracts. Values are expressed as a mean ± S.D. of 5 experiments with duplicate tubes (*P< 0.05 - compared to control erythrocytes with H_2O_2 alone. -: not determined)

Plant	Extract Conc.(mg/ml)	Alainine (nmol/g Hb)		MDA (nmol/g Hb)		IF	
		Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
Nigella sativa	0.0	435.6±189.5	3033.7±395	10±0.5	300.7±27.3	13.2±1.7	136.2±19.1
	0.2	-	2403.5±455.7*	-	263.6±32*	-	73.6±8.3*
	0.4	-	1777.5±425*	-	226.4±37*	-	43.6±5*
	0.6	-	1399.3+-386.7*	-	203.7±22*	-	33.7±5.5*
	0.8	-	943.8±26839*	8±1.7	197.8±28.7*	-	25.6±5.8*
	1.0	435.3±224	674±158*	-	-	13.0±2.2	19.2±3.8*
Allium sativum (%)	0.0%	445.8±20	4741.8±576	9.5±3.3	310±42	15.3±1.4	163.3±19.8
	25%	-	3790.3±246.7*	-	250±29*	-	130.9±18*
	50%	-	3055.4±714*	-	216.4±31.7*	-	98.7±10.2*
	100%	380±21	2621.6±399*	9±2.9	208.7±22*	13.1±0.7	75.0±5.7*
Ferula hermonis	0.0	513.7±66	3241.8±261.1	17.6±4.7	327±13	13.3±2.8	81.4±8.9
	0.8	-	3285±152	12.9±301	184.8±10*	14.5±304	95.6±25.6
Hibiscus sabdariffa	0.0	465.1±191	2817.9±434.9	9.9±4.4	381.2±24	13.0±2.1	80.0±11.3
	0.8	-	3027.3±564.5	9.9±3.3	255.4±14*	12.8±0.9	87.2±9.7
Teucrium polium	0.0	369.2±34	3352.8±262.7	17.6±3	363.6±68	9.1±1.0	66.5±10.6
	0.8	-	3378.7±145	12.9±3	236.7±38*	11.5±2.8	61.2±8.4
Trigonella foenum-graecum	0.0	398±40	3412.3±308	13.8±2.9	305.6±20	12.2±1.7	107.1±9.8
	0.8	-	3356.1±195	16±3.7	462.4±30*	12.7±0.8	104.6±7.3
Artemisia herba-alba	0.0	439.8±73	3322.8±284.6	17.6±4	327±13	14.3±3.0	76.2±4.7
	0.8	-	3300.7±217	12.9±3	327±13	15.3±2.5	76.0±2.8

Destruction of erythrocytes by oxidative mechanisms is known to be the end result of two closely related processes; namely, denaturation of Hb and oxidation of erythrocyte membrane. Exposure of erythrocytes to H_2O_2 has been observed to bring about lipid peroxidation, protein degradation and progressive loss of deformability in a concentration and time dependent manner [7,8]. The present work aimed to determine which of the detrimental effects of oxidative stress, namely, lipid peroxidation or protein degradation, is responsible for loss of erythrocyte deformability. For this purpose, different natural and synthetic antioxidants were tested for their protective effects on erythrocyte deformability, lipid peroxidation and protein degradation after exposure to H_2O_2. Antioxidants used included vitamin E, BHT, vitamin C, PNU-101033E, CO, and selected flavonoids and herbal extracts.

According to our previous studies [7,8], exposure of normal erythrocytes to 10 mM H_2O_2 caused a significant increase in IF (*i.e*. a significant loss of deformability). However, the present study also showed that lipid soluble antioxidants (vitamin E and BHT) were found to inhibit lipid peroxidation, with no protective effects against protein degradation or loss of erythrocyte deformability. Our results are in agreement with other studies which reported that BHT decreased lipid peroxidation without affecting proteolysis [5].

In the present study, vitamin C caused a significant increase in alanine production (i.e., protein degradation) consistent with a significant increase in IF values, namely, loss of erythrocyte deformability. Such increase in alanine production and IF values were not observed in erythrocytes that were pre-incubated with vitamin C but without being exposed to 10 mM H_2O_2. Vitamin C also provided more than 50% protection against MDA production (i.e., anti-lipid peroxidation) when added to erythrocytes before being exposed to H_2O_2. These findings, when taken together, suggest that vitamin C acts as a site-specific pro-oxidant toward proteins rather than to lipid. This pro-oxidant property of vitamin C may be attributed either to its ability to stimulate the redox cycling of free iron ions inside RBC cytosol released after the exposure to H_2O_2, or to its ability to stimulate the reaction between H_2O_2 and iron-moiety of Hb, enhancing the generation of various 'oxo-hemo-oxidant' or 'caged radicals' which are involved in Hb oxidation [4,5]. However, the specific mechanism by which vitamin C enhances protein degradation remains uncertain. From this result, it can be inferred that vitamin C, although it acts as antioxidant in one system, it does not necessarily act as antioxidant in other systems. If this is borne in mind, caution should be exercised in its therapeutic use especially under oxidant stress. In support of this suggestion, several studies have reported that administration of vitamin C to thalassemic patients had led to increased excretion of the oxidation product, oxalic acid, in the urine [17]. Also, vitamin C was found by others to increase lytic sensitivity of erythrocytes to H_2O_2 [18]. Moreover, high supplementation of vitamin C in diets of weanling rats was found to significantly increase the *in vitro* RBC hemolysis and liver peroxidation, also to significantly lower erythrocyte level of reduced glutathione (GSH) and plasma level of vitamin E [19]. Furthermore, when vitamin C was injected into rats, erythrocytes became more labile to H_2O_2 induced oxidative hemolysis [20]. Other studies also showed that supplementation of vitamin C to healthy, non-smoking males and females suppressed significantly the activities of antioxidant enzymes superoxide dismutase and glutathione peroxidase [21]. The present study therefore supports previous findings by others of possible adverse effects of vitamin C under oxidative stress [22].

PNU-101033E is a potent inhibitor of lipid peroxidation being developed by Pharmacia & Upjohn (Kalamazoo, MI, USA). PNU-101033E have been found by others to prevent completely the formation of toxic aldehydes, thereby inhibiting subsequent protein adducts formation, and cross-linking caused by these aldehydes [23,24]. In the present study, pre-incubation of erythrocytes with PNU-101033E alone or in the presence of H_2O_2 showed no effect on IF values or alanine production (Figs. 4), but it was found to decrease MDA production (*i.e*. inhibits lipid peroxidation) in a concentration dependent manner (Fig. 4).

In the present study, pre-incubation of erythrocytes with CO was found to prevent almost completely the loss of deformability caused by H_2O_2 (Fig. 5), while decreasing significantly MDA and alanine productions (Figs. 5). This result is in accordance with Snyder *et al*. [25] who found that CO could completely prevent hemoglobin degradation caused by oxidant stress. CO is known to bind hemoglobin (Hb) molecule tightly in the open coordination site of the heme-Fe^{2+}. The conversion of Hb to HbCO has previously been observed to inhibit its

peroxidation-promoting activity [7,16]. In addition, CO can provide protection against hyperoxic lung injury [26]. According to Snyder and his colleagues, CO can stabilize hemoglobin in the oxy configuration and block its function as an electron trap, so it prevents hemoglobin degradation or cross-linking with other proteins when exposed to H_2O_2 [25]. Hence, it seems likely that the fraction of lipid peroxidation that was prevented by CO was the part caused by the oxygen radicals released by the oxidation of hemoglobin.

In the present study, pre-incubation of erythrocytes with the flavonoids Quercetin and 3,5,7-trihydroxy-4'-methoxy flavone-7-rutinoside significantly protected erythrocytes against loss of erythrocyte deformability and protein degradation as compared with those treated with H_2O_2 alone (Table 1). In contrast, the flavonoids rutin and morin showed no protection against loss of erythrocyte deformability or protein degradation, despite of their protection against lipid peroxidation (Table 1). The protective activity of the flavonoid 3,5,7-trihydroxy-4'-methoxy flavone-7-rutinoside against loss of erythrocyte deformability appeared to be independent of lipid peroxidation since this flavonoid inhibited protein degradation without affecting lipid peroxidation (Table 1). The anti-lipid peroxidant activities of quercetin, rutin and morin were reported by others [27,28].

In the present study, pre-incubation of erythrocytes with the herbal extracts of *Ferula hermonis*, *Hibiscus sabdariffa*, *Teucrium polium* and *Trigonella foenum-graecum* showed no effects on protein degradation or erythrocyte deformability, although *Ferula hermonis*, *Hibiscus sabdariffa* and *Teucrium polium* protected against lipid peroxidation (Table 2). However, *Nigella sativa* and *Allium sativum* protected human erythrocytes against protein degradation and loss of deformability in a concentration dependent manner (Fig. 5). *Artemisia herba-alba* had no significant effect on either MDA production, protein degradation or erythrocyte deformability, *Trigonella foenum-graecum* although increased significantly lipid peroxidation, it did not have any effect on erythrocyte deformability (Table 2).

Considering the results of the present study, it seems noteworthy that protein oxidation with consequent degradation may prove to be of practical significance in the loss of erythrocyte deformability under oxidative stress. Lipid peroxidation however, does not appear to be responsible for the loss of erythrocyte deformability, since treatment of erythrocytes with the antioxidants that inhibited MDA production, were unable to prevent protein degradation or loss of deformability. Therefore, it can be concluded that, the loss of deformability of erythrocytes under oxidative stress is largely due to protein oxidation with consequent degradation rather than to lipid peroxidation. This loss of deformability appears to be related to oxidation of heme proteins resulting in their cross-linking to skeletal proteins (*i.e.*, spectrin and actin) and to the cytoplasmic component of band 3 [25,29]. These results are also compatible with Davies and Goldberg [4] conclusion, which states that lipid peroxidation and protein degradation occur by independent mechanisms.

5. CONCLUSION

1. Exposure of human erythrocytes to H_2O_2 causes lipid peroxidation, protein oxidation with consequent degradation and loss of deformability.
2. Loss of erythrocyte deformability under oxidative stress is largely due to protein oxidation with consequent degradation rather than to lipid peroxidation.
3. Lipid peroxidation and protein degradation occur by independent mechanisms, since some antioxidants can prevent one of them without the other.
4. Caution should be exercised in the therapeutic use of vitamin C, especially under oxidant stress.
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CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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